Basic Science

Ursodeoxycholic acid improves insulin sensitivity and hepatic steatosis by inducing the excretion of hepatic lipids in high-fat diet–fed KK-A\(^{y}\) mice

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Type 2 diabetes mellitus is frequently accompanied by fatty liver/nonalcoholic fatty liver disease. Hence, accumulation of lipids in the liver is considered to be one of the risk factors for insulin resistance and metabolic syndrome. Ursodeoxycholic acid (UDCA) is widely used for the treatment of liver dysfunction. We investigated the therapeutic effects of UDCA on type 2 diabetes mellitus exacerbating hepatic steatosis and the underlying mechanisms of its action using KK-A\(^{y}\) mice fed a high-fat diet. KK-A\(^{y}\) mice were prefed a high-fat diet; and 50, 150, and 450 mg/kg of UDCA was orally administered for 2 or 3 weeks. Administration of UDCA decreased fasting hyperglycemia and hyperinsulinemia. Hyperinsulinemic-euglycemic clamp analyses showed that UDCA improved hepatic (but not peripheral) insulin resistance. Hepatic triglyceride and cholesterol contents were significantly reduced by treatment with UDCA, although the genes involved in the synthesis of fatty acids and cholesterol, including fatty acid synthase and 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, were upregulated. Fecal levels of bile acids, neutral sterols, fatty acids, and phospholipids were significantly increased by UDCA treatment. The gene expression levels and protein phosphorylation levels of endoplasmic reticulum stress markers were not changed by UDCA treatment. These results indicate that UDCA ameliorates hyperglycemia and hyperinsulinemia by improving hepatic insulin resistance and steatosis in high-fat diet–fed KK-A\(^{y}\) mice. Reduction of hepatic lipids might be due to their excretion in feces, followed by enhanced utilization of glucose for the synthesis of fatty acids and cholesterol. Ursodeoxycholic acid should be effective for the treatment of type 2 diabetes mellitus accompanying hepatic steatosis.

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1. Introduction

Hepatic steatosis is a major element of nonalcoholic fatty liver disease (NAFLD), which affects approximately 20% of the adult population and is found in more than two thirds of obese people [1]. Up to 70% to 80% of individuals with NAFLD have insulin resistance or metabolic syndrome [2]. Epidemiological data have shown that fatty liver is an independent prognostic factor of type 2 diabetes mellitus [3-7]. Weight reduction by lifestyle modification is generally recommended for the treatment of NAFLD [8-10].

For many years, ursodeoxycholic acid (UDCA) has been widely used in the treatment of cholestatic liver diseases, including primary biliary cirrhosis and primary sclerosing cholangitis. Recent reports indicate that UDCA and taurine-conjugated UDCA (TUDCA) improved glucose metabolism; that is, administration of high-dose UDCA improved glycemic parameters and insulin resistance surrogate markers in patients with nonalcoholic steatohepatitis [11], and TUDCA increased insulin sensitivity in obese, insulin-resistant subjects [12]. Although TUDCA has been reported to increase insulin sensitivity in ob/ob mice by ameliorating endoplasmic reticulum (ER) stress [13], it improved hepatic and muscular insulin sensitivity in obese subjects without changing indicators of ER stress [12]. Therefore, the underlying mechanisms of insulin-sensitizing action of UDCA and TUDCA remain unclear.

In this study, we examined the antidiabetic effect of UDCA in high-fat diet–fed KK-Ay mice, which showed exacerbation of hepatic steatosis and insulin resistance. We also investigated the underlying mechanism of action, particularly focusing on the gene expression levels involved in the lipid metabolism in the liver as well as lipid contents and their composition in feces.

![Graphs showing effect of UDCA on fasting plasma glucose, insulin, HOMA-IR, food intake, and body weight.](image)

**Fig. 1** – Effect of UDCA on fasting plasma glucose, insulin, HOMA-IR, food intake, and body weight. Plasma glucose (A) and plasma insulin (B) were measured, and HOMA-IR (C) was calculated on day 13 after overnight fasting in KK-Ay mice fed a high-fat diet. Food intake (D) between day 1 and day 12 was measured. Body weights (E) on day 12 were measured. Data are expressed as means ± SEM (n = 5). *P < .05, **P < .01 vs high-fat diet control. ††P < .01 vs normal diet.
2. Methods

2.1. Chemicals

Ursodeoxycholic acid was synthesized at Mitsubishi Tanabe Pharma Corp. (Osaka, Japan) and dissolved in 1 mol/L NaOH followed by adjusting to pH 8.3 with HCl for oral administration as described previously [14].

2.2. Animals and diets

Male KK-Ay mice were purchased from CLEA (Tokyo, Japan). Mice were housed individually and maintained under a constant 12-hour light/dark cycle with free access to water and the diets described below. They were allowed to acclimatize for 5 to 6 weeks. A purified-ingredient, high-fat diet with 45 kcal% fat primarily from lard (#D12451, Research Diets, New Brunswick, NJ) was used to accelerate insulin resistance and hepatic steatosis. A 10 kcal% fat diet (#D12450B, Research Diets) was used as the normal diet. Animal experiments were approved by the Institutional Animal Care and Use Committee of Mitsubishi Tanabe Pharma Corp.

2.3. Experimental protocol

After acclimatization, mice were divided into experimental groups matched for body weight, plasma levels of glucose, and insulin. Indicated doses of UDCA were orally administered to mice at 5 mL/kg body weight once a day for 2 or 3 weeks. Purified water was administered to the high-fat diet control and normal-diet groups. Plasma levels of glucose and insulin were determined by a glucose assay reagent (Wako, Osaka, Japan) and an enzyme-linked immunosorbent assay kit (Shibayagi, Gunma, Japan), respectively. Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated [15]. After the final administration, all mice were anesthetized and then killed by the collection of blood from the abdominal vein. Livers were immediately removed and stored at −80°C for subsequent determination of lipid parameters, quantitative polymerase chain reaction (PCR), and phosphorylation levels of c-Jun.

2.4. Hyperinsulinemic-euglycemic clamp

Different groups of mice were used for these experiments. Hyperinsulinemic-euglycemic clamp was performed at week 3 as described previously [16,17]. In brief, after fasting overnight, the hyperinsulinemic condition was started with a bolus (19 mU) followed by continuous infusion of insulin (32 mU/h) and of [3H]-glucose (1.2 μCi/h). A variable infusion of 12.5% unlabeled glucose solution was applied to maintain euglycemia, as measured in blood samples (every 10 minutes) taken by tail vein. At 70, 80, and 90 minutes after the start of the insulin infusion, blood samples were drawn to determine glucose concentration and disintegrations per minute of [3H]-glucose. Additional samples were taken to determine insulin at

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Fig. 2 – Effect of UDCA on insulin sensitivity by hyperinsulinemic-euglycemic clamp study. After treatment with 150 mg/kg UDCA for 3 weeks, GIR (A), HGP (B), and BGU (C) were determined by hyperinsulinemic-euglycemic clamp analysis after an overnight fast. Data are expressed as means ± SEM (n = 8–9). **P < .01 vs high-fat diet control. ††P < .01 vs normal diet.
2.5. Determination of hepatic levels of triglyceride and total cholesterol

Hepatic lipids were extracted using chloroform-methanol (1:1 by volume) according to the method suggested by Folch et al [18]. Levels of triglyceride and total cholesterol were determined using the Triglyceride E-Test Wako and Cholesterol E-Test Wako assay reagents, respectively.

2.6. Determination of hepatic glycogen levels

Hepatic glycogen was extracted and assayed according to the method suggested by Farquharson et al [19] with slight modifications. Glucose levels in the liver lysate were determined by a glucose assay reagent (Wako).

2.7. Quantitative PCR

The total RNA was extracted from the livers and reverse-transcribed using the TaqMan Reverse Transcriptase Reagents (Applied Biosystems, Foster City, CA). Real-time PCR was performed with SYBR Green PCR mix (Applied Biosystems) and analyzed with ABI Prism 7000 Sequence Detection System (Applied Biosystems). Relative expression levels were compared after normalization to β-actin. β-Actin primer was from Qiagen (Tokyo, Japan). The following primer pairs were used (5′ to 3′): Cpt-1a, forward: AAGCTGTTCAAGATAGCTTG, reverse: TGCTGATGACGGCTATGGTGT; Acox1, forward: GGTTAAAGGTGCTTTCTGCC, reverse: AGATAAACTCCCCAA-GATTCAAGAC; Fas, forward: GAACCTGAGGGTCTCTACCC, reverse: CAAGGAACAGAGCGGCTC; Hmgcr, forward: CCGGAAACAAGAAGATCCTGTG, reverse: ATGTACAGGATGGC-GATGCA; Acl, forward: CAGCCAAGGCAATTTCAGAGC, reverse: CTGCGATGTTTGATTAACTGGTCT; Hmgcs, forward: GTGGCACCGGATGTCTTTG, reverse: ACTCTGACCAGATAC- CAGTT; Acc2, forward: GGCTCTTGGGTGACGATAAC; Pepck, forward: GCTCTGAGGAGAATGG, reverse: TGCTCTTGGGTGACGATAAC; G6pase, forward: GGATCCTGGGACAGACACAA, reverse: GTCCGGAGGAGTTCTGGA; Xbp-1, forward: ACCGGAGCATTCAGAGC, reverse: GCCGCGCGGATGTCTTTG; Bnip3, forward: TTCACTACTCTTT-CAGTTGCTCC, reverse: CACTGACCACCTCTGTTCCGTTTC.

2.8. Collection of feces and fecal lipid measurements

The cage bedding including feces over a period of 2 days was collected at week 2. Feces were separated from the cage bedding and subsequently lyophilized and weighed. Fecal lipids were extracted from an aliquot of feces. Fecal fatty acids, sterols, and bile acids were determined by gas chromatography as described previously [20]. Phospholipid concentrations were measured by a commercially available phospholipid assay kit (Instruchemie, Delfzijl, the Netherlands).

2.9. Determination of phosphorylation levels of c-Jun

Lysates were prepared using lysis buffer (Cell Signaling, Tokyo, Japan). Phosphorylation levels of c-Jun were determined by Bio-Plex Phosphoprotein Detection Reagent Kit (Bio-Rad, Tokyo, Japan) and Bio-Plex Phospho-c-Jun (Ser63) Assay (Bio-Rad) according to the manufacturer’s protocol as described previously [21]. The total proteins for c-Jun were measured by Bio-Plex Total c-Jun Assay (Bio-Rad). Data were acquired and analyzed using the Bio-Plex suspension array system (Luminex 100 system) from Bio-Rad Laboratories. Phosphorylation index was calculated as phospho-c-Jun (Ser63) divided by total c-Jun.

2.10. Statistical analyses

All data are presented as means ± SEM. Comparison of mean values between 2 groups was evaluated by the Student t test. We used the Dunnett test for comparing more than 2 groups. P values < .05 were considered statistically significant.

3. Results

3.1. Effect on fasting glucose, insulin, HOMA-IR, food intake, and body weight

KK-Ay mice were prefed a high-fat diet; and 50, 150, and 450 mg/kg UDCA was orally administered. Compared with the...
normal diet control, the high-fat diet control showed higher levels of fasting glucose, insulin, and HOMA-IR (Fig. 1A-C), indicating exacerbation of insulin sensitivity. Administration of UDCA resulted in a dose-dependent reduction of these parameters (Fig. 1A-C). The UDCA treatment did not change food intake and body weight (Fig. 1D, E). In a subsequent study, we used 150 mg/kg UDCA to evaluate the underlying mechanism of its action because HOMA-IR, which is an index of insulin sensitivity, was significantly improved by 150 mg/kg.

3.2. Effect on insulin sensitivity

To further investigate the effect of UDCA on insulin sensitivity, we performed hyperinsulinemic-euglycemic clamp analyses. We confirmed that the steady-state insulin levels at 90 minutes after the start of the insulin infusion were hyperinsulinemia in 3 groups (30.3 ± 4.6, 42.4 ± 6.7, and 37.8 ± 5.8 ng/mL in normal diet group, high-fat diet group, and UDCA treatment group, respectively). A high-fat diet induced a lower GIR, higher HGP, and lower BGU than a normal diet, indicating hepatic and peripheral insulin resistance (Fig. 2A-C). The UDCA treatment improved the GIR (Fig. 2A) and HGP (Fig. 2B) but did not affect BGU (Fig. 2C). These data suggested that UDCA improved mainly hepatic (but not peripheral) insulin resistance.

3.3. Effect on hepatic steatosis

Next, we examined the effect of UDCA on hepatic steatosis. Higher hepatic triglyceride and cholesterol contents were

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**Fig. 4** – Effect of UDCA on hepatic gene expression. After treatment with 150 mg/kg UDCA for 2 weeks, RNA was extracted from the liver; and hepatic mRNA levels of genes involved in β-oxidation (A), fatty acid synthesis (B), and cholesterol synthesis (C) were measured. Data are expressed as means ± SEM (n = 7). *P < .05, **P < .01 vs high-fat diet control. †P < .05, ††P < .01 vs normal diet.
observed in mice fed a high-fat diet (Fig. 3A, B). The UDCA treatment significantly reduced both these parameters (Fig. 3A, B).

3.4. Effect on lipid metabolism–related genes in the liver

To understand the underlying mechanism of hepatic lipid reduction by UDCA, we measured expression levels of genes involved in β-oxidation (Cpt-1a, Acox1), and in the synthesis of fatty acids (Fas, Acl, Acc2) and cholesterol (Hmgcs, Hmgcr). Genes involved in β-oxidation were unchanged between groups (Fig. 4A). Fatty acid and cholesterol synthesis–related genes were downregulated by feeding of a high-fat diet (Fig. 4B, C). Surprisingly, UDCA treatment significantly upregulated their expression levels (Fig. 4B, C). These data suggested that UDCA might increase the synthesis of fatty acids and cholesterol in the livers of KK-Ay mice fed a high-fat diet.

3.5. Effect on fecal excretion of bile acids, neutral sterols, fatty acids, and phospholipids

From the above data, we hypothesized that UDCA enhanced excretion of newly synthesized hepatic lipids. To confirm our working hypothesis, we measured fecal lipid content and composition. As expected, UDCA treatment led to a massive increase in fecal bile acids, neutral sterols, fatty acids, and phospholipids (Fig. 5A-D). Changes in fecal neutral sterol and fatty acid composition were also observed. The ratio of cholesterol was significantly increased, whereas that of coprostanol and cholestanol was decreased (Table 1A). Among neutral sterols, cholesterol was synthesized in the liver, whereas coprostanol and cholestanol were cholesterol metabolites in the gut. Furthermore, the ratio of C18:1 fatty acid, which was synthesized by a de novo pathway and abundantly present in the liver, was increased (Table 1B). These results suggested that UDCA might induce the excretion of hepatic lipids into feces, which contributed to a improvement of hepatic steatosis.

3.6. Effect on gluconeogenesis-associated genes and glycogen contents in the liver

To understand the detailed mechanism of hypoglycemic effect of UDCA, we measured the gene expression levels of gluconeogenesis–associated enzymes and glycogen contents in the liver. Gene expression levels of G6Pase and Pepck were not changed (Fig. 6A), whereas hepatic contents of glycogen tended to be increased (but not statistically significant) by UDCA treatment (Fig. 6B).

3.7. Effect on ER stress markers in the liver

Finally, we examined the effect of UDCA on ER stress markers in the liver. Gene expression levels of Xbp-1, Bip,
and Chop (Fig. 7A), and JNK activity indicated by phosphorylated levels of c-Jun (Fig. 7B) were not changed by UDCA treatment. These results suggested that UDCA improved hepatic insulin resistance without affecting ER stress in our model.

### 4. Discussion

Fatty liver is reported to be closely associated with insulin resistance and type 2 diabetes mellitus [3-7]. In the present studies...
study, to clarify the effects of UDCA on insulin resistance and fatty liver, we used KK-A^Y mice fed a high-fat diet because they showed severe hyperglycemia, hyperinsulinemia, and hepatic steatosis compared with KK-A^Y mice fed a normal diet.

Hyperinsulinemic-euglycemic clamp studies revealed that a high-fat diet exacerbated hepatic and peripheral insulin sensitivity. UDCA improved HGP, but not BGU. These results indicated that UDCA mainly improved hepatic insulin resistance. Decreased HGP might be due to inhibition of glycogenolysis but not gluconeogenesis because hepatic levels of glycogen were increased and gene expression levels of G6Pase and Pepck were not changed by UDCA treatment. Improvement of hepatic insulin resistance by UDCA resulted in the reduction of hyperglycemia and hyperinsulinemia.

Furthermore, UDCA significantly reduced the hepatic contents of triglyceride and cholesterol. An inverse relationship between hepatic triglyceride and hepatic insulin sensitivity has been reported [22], so it is reasonable to suggest that UDCA improved hepatic insulin resistance with decreasing levels of hepatic lipids.

Inconsistent with decreasing hepatic levels of triglyceride and cholesterol, UDCA upregulated genes encoding for the fatty acid (Fas, Acl, Acc2) and cholesterol (Hmgcs, Hmgcr) synthesis-related enzymes, whereas genes involved in β-oxidation (Cpt-1a, Acox1) in the liver were unchanged. UDCA has been reported to induce the activation of bile acid efflux transporters such as Mrp2, Mrp3, and Mrp4 in the liver [23]; so we analyzed lipid contents and composition in feces. Fecal contents of bile acids, neutral sterols, fatty acids, and phospholipids were massively increased by UDCA treatment. Therefore, we speculate that UDCA might induce excretion of hepatic lipids into feces, which resulted in compensatory upregulation of genes involved in the synthesis of fatty acids and cholesterol, leading to activation of the metabolism from glucose to lipids in the liver (Fig. 8).

Fecal neutral sterol and fatty acid composition data support this hypothesis. First, UDCA treatment increased the ratio of cholesterol, which was synthesized in the liver, and decreased that of cholesterol metabolites in the gut (eg, coprostanol, cholestanol). Second, the ratio of C18:1 fatty acid was significantly increased. C18:1 fatty acid is synthesized by a de novo
pathway and is abundantly present in the liver. These results suggested that fecal neutral sterols and fatty acids were also considered to be mainly derived from the liver. Furthermore, increased fecal excretion of C18:1 fatty acid may be related to increased hepatic insulin sensitivity because it was reported that mice deficient for Elov6, the gene encoding the elongase that catalyzes the conversion of C16 to C18, were protected from obesity-induced insulin resistance [24]. It should be stressed that this hypothesis requires further investigation.

Although TUDCA has been reported to alleviate ER stress and improve systemic insulin resistance in ob/ob mice [13], UDCA improved only hepatic insulin sensitivity without affecting ER stress in our study. This difference could be explained by the animal models. ER stress levels were reported to be elevated in ob/ob mice compared with those in normal mice [13]. We also confirmed that ER stress levels in the liver and peripheral tissues of ob/ob mice were higher than those of ob/+ mice (data not shown). On the other hand, gene expression levels of ER stress markers and phosphorylation levels of c-Jun in the liver of high-fat diet-fed KK-A^y^ mice were comparable to those of normal diet-fed KK-A^y^, indicating that ER stress is not involved in the high-fat diet-induced hepatic insulin resistance of KK-A^y^ mice. It is likely that TUDCA restored hepatic and peripheral insulin sensitivity by ameliorating ER stress in ob/ob mice, whereas UDCA improved hepatic insulin resistance by inducing the excretion of hepatic lipids without affecting ER stress in our model.

Farnesoid X receptor (FXR) is a nuclear receptor that binds various bile acids, and FXR agonists have been reported to improve glucose metabolism by repressing the expression levels of hepatic gluconeogenic enzymes [25]. UDCA is reported to be a weak ligand of FXR [26]; and expression levels of Shp mRNA, a well-characterized FXR target gene [25], were unchanged by UDCA treatment in our study (data not shown). Therefore, UDCA might improve insulin sensitivity without affecting FXR activity.

In this study, we used mainly 150 mg/kg of UDCA, which is considered to be the clinical dose of 600 mg per man per day for the treatment of cholestatic liver diseases [14,27]. On the other hand, it was reported that high-dose UDCA (28-35 mg/[kg d]) improved glycemic parameters including serum glucose, hemoglobin A1c, and insulin in patients with nonalcoholic steatohepatitis [11]. The difference between estimated dose (600 mg per man per day) from animal models and reported clinical dose (28-35 mg/[kg d]) of UDCA on hypoglycemic effect remains unclear, and further information or clinical studies are needed.

In summary, we have demonstrated that UDCA improved insulin resistance and hepatic steatosis in KK-A^y^ mice fed a high-fat diet by accelerating the efflux of lipids from the liver into feces. Given its well-established safety in humans, UDCA should be a novel approach for the treatment of type 2 diabetes mellitus showing to exacerbate hepatic steatosis.

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Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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